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Expression and Characterization of Human Recombinant and α-*N*-Actylglucosaminidase

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Mucopolysaccharidosis type IIIB (MPS-IIIB, Sanfilippo type B Syndrome) is a heterosomal, recessive lysosomal storage disorder resulting from a deficiency of [alpha]-N-acetylglucosaminidase (NAGLU). To characterize this enzyme further and evaluate its potential for enzyme replacement studies we expressed the NAGLU-encoding cDNA in Chinese hamster ovary cells (CHO-K1 cells) and purified the recombinant enzyme from the medium of stably transfected cells by a two-step affinity chromatography. Two isoforms of recombinant NAGLU with apparent molecular weights of 89 and 79 kDa were purified and shown to differ in their glycosylation pattern. The catalytic parameters of both forms of the recombinant enzyme were indistinguishable from each other and similar to those of NAGLU purified from various tissues. However, compared to other recombinant lysosomal enzymes expressed from CHO-K1 cells, the mannose-6-phosphate receptor mediated uptake of the secreted form of recombinant NAGLU into cultured skin fibroblasts was considerably reduced. A small amount of phosphorylated NAGLU present in purified enzyme preparations was shown to be endocytosed by MPS-IIIB fibroblasts via the mannose-6-phosphate receptor-mediated pathway and transported to the lysosomes, where they corrected the storage phenotype. Direct metabolic labeling experiments with $Na_2{}^{32}PO_4$ confirmed that the specific phosphorylation of recombinant NAGLU secreted from transfected CHO cells is significantly lower when compared with a control lysosomal enzyme. These results suggest that the use of secreted NAGLU in future enzyme and gene replacement therapy protocols will be severely limited due to its small degree of mannose-6-phosphorylation. © 2001 Academic Press

Key Words: **mucopolysaccharidosis; Sanfilippo syndrome; α-*N*-acetylglucosaminidase; lysosomal degradation; heparan sulfate; intracellular transport; phosphorylation.**

α-*N*-Acetylglucosaminidase (NAGLU,[3] EC 3.2.1.50) is one of a number of lysosomal exoenzymes involved in the degradation of heparan sulfate. A lack of active NAGLU results in the lysosomal storage disorder mucopolysaccharidosis III type B (MPS-IIIB) one of the four subtypes of Sanfilippo syndrome. Taken together the Sanfilippo subtypes have an incidence of about 1 in 24,000 births (1). A lower incidence of 1 in 66,000 has been reported in Australia (2). Patients present with severe, progressive neurodegeneration resulting in mental retardation and often death in their teens or early twenties (3). First symptoms in profoundly affected patients include delayed psychomotor and speech development often noticed by the age of 2 while in patients with delayed onset the rapid loss of social skills, like hyperactive and aggressive behavior, is one of the first symptoms. The typical facial features that characterize other MPS disorders are very mild or absent in

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[3] Abbreviations used: CHO-K1, Chinese hamster ovary cells; Coons/DMEM, Coon's Dulbecco's modified Eagle medium; FCS, fetal calf serum; GAA, lysosomal acid α-glucosidase; GAG, glycosaminoglycan; GlcNac-IdOA, *O*-(α-acetamido-2-deoxy-D-glucopyranosyl)-(1–3)-[L-6,^{3}H]idoronic acid; I2S, iduronate-2-sulfatase; M6P, mannose-6-phosphate; MPR, mannose-6-phosphate receptor; MPS-IIIB, mucopolysaccharidosis III type B or Sanfilippo syndrome type B; NaAc, sodium acetate; NAGLU, α-*N*-acetylglucosaminidase; PNGase F, *N*-glucosidase F; rNAGLU, recombinant α-*N*-acetylglucosaminidase; rf4S, recombinant feline *N*-acetylgalactosamine 4-sulfatase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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Sanfilippo patients and, together with a high rate of false negative results by some tests for heparan sulfaturia, diagnosis is often delayed (3).

NAGLU acts as an exoglycosidase on the nonreducing-end of α-*N*-acetylglucosaminide residues of heparan sulfate (4) and is one of the three lysosomal enzymes involved exclusively in the degradation of this glycosaminoglycan (GAG). Native NAGLU has been purified partially (5–8) and to apparent homogeneity (9–11) from a number of different tissues. The native enzyme purified from human placenta shows an apparent molecular weight of 80 kDa for the precursor and 77 kDa for the mature form. cDNA clones isolated by different groups using peptide sequence information (10, 11) predict a molecular mass of 82 kDa (743 amino acids), including a signal peptide of 23 amino acids and revealed seven potential glycosylation sites. From the differences in apparent molecular mass of the native protein and the mass predicted by the NAGLU amino acid sequence, it is estimated that the enzyme carries one or two carbohydrate side chains. This probably includes the site at residue 272 of the amino acid sequence since N-terminal sequencing of a CNBr peptide was blocked at this position (10). As the majority of soluble lysosomal enzymes are transported via the mannose-6-phosphate receptor (MPR)-mediated pathway (12), at least one of the glycosylation sites is expected to be carrying a phosphorylated carbohydrate side chain. Studies involving other recombinant lysosomal enzymes showed that Chinese hamster ovary (CHO-K1) cells are capable of producing correctly post-translational modified protein which was competent in uptake and correction studies (13–16) implying the generation of phosphorylated mannose residues.

The NAGLU gene is located on chromosome 17*q*21.1 and its structure is characterized (10, 11), which allowed several studies of the genetic causes of MPS-IIIB (11, 17–20). As expected from the wide clinical variability that MPS-IIIB displays, a high degree of molecular heterogeneity was discovered recently with around 70 different mutations ranging from small deletions/insertions to nonsense and missense mutations identified to date. One of the missense mutations (R643C) is clearly associated with the attenuated form of MPS-IIIB (20), whereas the effect of a nonpathogenic sequence variation (G737R) (20) has not yet been characterized.

This study aims to provide a more detailed understanding of the properties of recombinant NAGLU (rNAGLU) as a basis for the functional analysis of mutant NAGLU expressed in cell culture systems or patient fibroblasts. The functional data obtained so far showed that although the enzymatic properties of rNAGLU are comparable to those of the native enzyme it appears that the secreted form of the recombinant enzyme is phosphorylated to a much lesser extent than seen with other recombinant lysosomal enzymes. It is likely but remains to be proven whether this low level of phosphorylation is also true for the secreted form of the native enzyme.

MATERIALS AND METHODS

Expression of rNAGLU

The cDNA encoding NAGLU was subcloned into the expression vector pcDNA3 (Stratagene) as described elsewhere (10). CHO-K1 cells were transfected with the construct using the DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer's instruction. After transfection cells were grown in Ham's F12 medium, 10% (v/v) fetal calf serum (FCS), penicillin, and streptomycin sulfate at 100 μg/mL each for 48 h and then incubated in medium containing 750 μg/mL G418 sulfate (Geniticin) until resistant colonies emerged.

Single cell clones were selected and 26 separate clones tested for expression of rNAGLU by measuring enzyme activity with the fluorogenic substrate.

Large-Scale rNAGLU Production

Cytodex 2 microcarrier beads (2 g) were swollen in 250 mL of PBS for 3 h at 37°C with three changes of PBS and then autoclaved for 15 min at 120°C (wet cycle). The beads were then rinsed with sterile growth medium (Coons/DMEM, 10% (v/v) FCS, penicillin, and streptomycin sulfate at 100 μg/ml each and 0.1% (w/v) Pluronic F68) and transferred into a Techne stirrer culture flask. The microcarrier beads were inoculated with seven confluent T75 flasks of the cell clone with the highest expression of rNAGLU. Growth medium (200 mL) was added and the culture incubated with a stirrer speed of 20 rpm to achieve an even distribution of cells on the beads. The cells were allowed to attach to the beads for 16 h at low speed. Growth medium was added to increase the total volume to 500 mL and the stirrer speed increased to 30 rpm. After a growth phase of 48 to 72 h with daily aerating to allow gas exchange the beads were completely covered with cells and the medium was exchanged for production medium (Coons/DMEM, without FCS, penicillin, and streptomycin sulfate at 100 μg/mL each, 0.1% (w/v) Pluronic F68, and 5 mM NH_4Cl). The glucose concentration was monitored daily and the medium replaced when glucose fell below 5 mM every 2–3 days. The harvested medium contained approximately 2 mg rNAGLU/L production medium.

Purification of rNAGLU

Production medium was dialyzed against 50 mM Na acetate, pH 5.5, and loaded onto a Heparin-Agarose

TABLE 1

Purification of Recombinant NAGLU

Purification step	Sp act (nmol/min/mg)	Recovery (%)	Purification (fold)
Dialyzed cell culture medium	6.59	100	1
Heparin-Agarose	72.5	68	11
DEAE-Sephacel (50 mM NaCl)	1060	10	161
DEAE-Sephacel (75 mM NaCl)	1057	8	160

(Sigma) column equilibrated in the same buffer. After washing with Na acetate buffer and Na acetate/50 mM NaCl the column was eluted with 75 mM NaCl in Na acetate buffer. The eluate was dialyzed against 20 mM Tris/HCl, pH 7.5, loaded onto a DEAE Sephacel column, washed with 25 mM NaCl in 20 mM Tris/HCl, and then eluted with 50 and 75 mM NaCl in 20 mM Tris/HCl, respectively. Results of the purification procedure are summarized in Table 1.

SDS-PAGE of the two eluates showed two bands associated with enzyme activity with apparent molecular weights of 79 and 89 kDa. The smaller form of rNAGLU was eluted predominantly in the 50 mM NaCl fraction, whereas the 89-kDa form was enriched in the 75 mM NaCl fraction (Fig. 1).

Enzyme Activity

Activity of rNAGLU was monitored with the fluorogenic substrate 4-methylumbelliferyl 2-acetamido-2-deoxy-α-D-glucopyranoside (21) (Calbiochem) as described (22). Briefly, samples were incubated with 1 mM substrate in 50 mM Na acetate buffer, pH 4.5, for 60 min at 37°C before fluorescence was determined. For determination of the pH optimum the assay was performed in 100 mM DMG buffer, pH 2.0 to 7.5.

A ^{3}H-labeled disaccharide GlcNAc-IdOA substrate, with a similar structure to the natural substrate heparan sulfate (23), was also used. The assay was performed in 100 mM DMG buffer, pH ranging from 3.7 to 5.6, with 60 μM GlcNAc-IdOA or in 100 mM DMG, pH 4.1, with the substrate concentration ranging from 180 to 5 μM. Substrate and product were separated by high-voltage electrophoresis on Whatman 3MM paper in 50 mM Na acetate, pH 5.5, at 3000 V for 50 min, the paper strips were scanned, and radioactivity in the two peaks was solubilized in scintillation fluid and counted with a standard tritium program.

Deglycosylation with PNGase F

Purified rNAGLU (20 μl) containing 2.5 and 3.8 μg of protein, respectively, were denatured by incubation with 50 μL 0.1 M β-mercaptoethanol/0.5% (w/v) SDS at 100°C for 5 min. Deglycosylation was performed with 2 U of PNGase F for 15 h at 37°C in 15 mM Tris/HCl, pH 8.0, 20 mM EDTA, 1.25% NP40. The control contained Tris buffer instead of PNGase F and was incubated under the same conditions. Deglycosylated rNAGLU and the controls were precipitated with trichloroacetic acid, run on SDS-PAGE (24), and stained with silver (25).

Immunization of Rabbits for Polyclonal Antisera Production

Rabbits were immunized using 1 mg of purified rNAGLU in five boosts. The preparation contained 576 μg of the fraction enriched in the 89-kDa form and 424 μg of the fraction enriched in the 79-kDa form. Antibody titers were tested by a standard ELISA procedure.

Metabolic Labeling of Recombinant Feline N-Acetylgalactosamine-4-sulfatase and rNAGLU

G418-resistant mass cultures of CHO-KI cells overexpressing recombinant feline *N*-acetylgalactosamine-4-sulfatase (rf4S) or rNAGLU were grown to confluence in 75 cm^2 flasks in Ham's F12 supplemented with FCS. Prior to metabolic labeling two 75 cm^2 flasks of each mass-culture were incubated with phosphate-free RPMI or cysteine-methionine-free DMEM (ICN Biomedicals Inc.) supplemented with L-glutamine and FCS for 1 h. Cells were then metabolically labeled with 0.1 mCi/mL $Na_2{}^{32}PO_4$ or 100 μCi/mL EXPRE $^{35}S^{35}S$ protein labeling mix (1175 Ci/mmol, DuPont, NEN) in FCS supplemented cysteine-methionine-free DMEM for 24 h. In some instances the metabolic labeling was performed in the presence of 5 mM M6P to prevent reuptake of

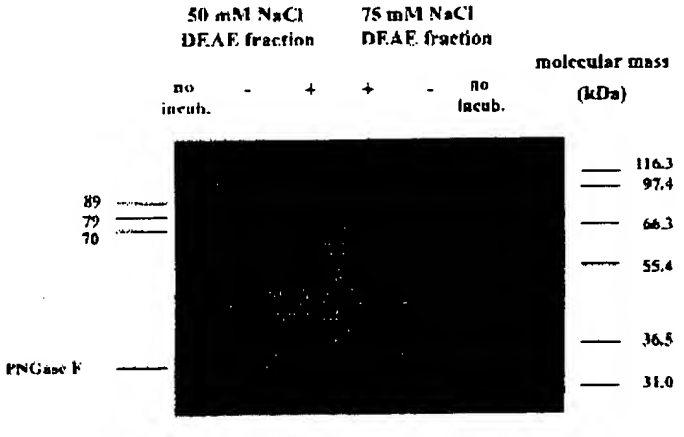

FIG. 1. SDS-PAGE of rNAGLU purified by ion-exchange chromatography before and after deglycosylation. Fractions enriched in either the 79-kDa or the 89-kDa form were separated by SDS-PAGE untreated (outer lanes 1 and 6), after control incubation without PNGaseF (lanes 2 and 5) and after incubation with 2 U of PNGaseF (lanes 3 and 4). The gel was stained with silver.

secreted enzymes. The medium which contained secreted $Na_2{}^{32}PO_4$- or ^{35}S-labeled recombinant enzymes was then harvested and clarified by centrifugation at 1500 rpm at 4°C for 5 min.

Uptake of ^{35}S-Labeled rf4S and ^{35}S-Labeled rNAGLU by Skin Fibroblasts

Control skin fibroblast cultures were grown to confluence in 75 cm^2 flasks in DMEM supplemented with FCS. The growth medium was removed and replaced with 7 mL of ^{35}S-labeled rf4S clarified medium or ^{35}S-labeled rNAGLU clarified medium (see above) in the presence or absence of 5 mM mannose-6-phosphate (M6P) and incubated for 48 h. Cells were harvested by treatment with trypsin-versene, recovered by centrifugation (1500*g* for 5 min), resuspended in 10 mL PBS, and then centrifuged again. The cell pellet was resuspended in 6 mL of solubilization buffer (PBS containing 1% w/v sodium deoxycholate, 0.1% (w/v) SDS, 0.5% Nonidet P-40) and incubated for 24 h at 4°C. Growth medium was also harvested, clarified by centrifugation, and used for immunoprecipitation (see below).

Immunoprecipitation

Prior to immunoprecipitation with rNAGLU polyclonal antisera or rf4S polyclonal antisera, growth medium and solubilized cell lysates were precleared as follows. Seventy microliters of polyclonal CHO-KI antiserum (obtained from CSL Ltd.) was added to each medium or solubilized lysate sample and incubated at 4°C overnight. Pansorbin cells (Calbiochem; 1.5 mL) were equilibrated by five washes in 1 mL of solubilization buffer by microcentrifugation/resuspension. Equilibrated Pansorbin cells (140 μL) were added to each sample, incubated at 4°C for a further 6 h, and then sedimented by microcentrifugation. Unpurified rabbit αrf4S serum (30 μl) (26) or unpurified rabbit αrNAGLU serum (30 μL, see above) was added to each supernatant and incubated at 4°C for 24 h. Pansorbin cells (125 μL) were added to each sample, incubated at 4°C for a further 24 h and then sedimented by centrifugation. The pellets, which contained the rf4S-antibody–protein A complexes or rNAGLU-antibody–protein A complexes, were washed four times in 1 mL of solubilization buffer by microcentrifugation/resuspension, once in 1 mL of distilled water and then resuspended in 50 μL sample buffer (1% (w/v) SDS, 4 M urea, 80 mM Tris–HCl, pH 6.8, 0.1% (w/v) Bromophenol blue). Samples were boiled for 5 min, centrifuged at 12,000 *g* for 1 min to pellet Pansorbin cells, and the supernatant analyzed via SDS-PAGE and autoradiography.

Uptake of rNAGLU and Correction of GAG Storage in MPS-IIIB Skin Fibroblasts

Analysis of GAG storage in cultured primary MPS-IIIB skin fibroblasts was determined using the method described by Harper *et al.* (27) with the following modifications. Control or MPS-IIIB (R297X/R297X homozygous) skin fibroblasts were grown to confluence in 75 cm^2 flasks in DMEM supplemented with FCS. Cells were pulse-labeled with 15 μCi/mL $Na_2{}^{35}SO_4$ (543 mCi/mmol; DuPont NEN) for 24 h in antibiotic-free F12 medium supplemented with 10% (v/v) FCS. Labeled cells were then washed with PBS, harvested with trypsin-versene (CSL Ltd.), and transferred to new flasks containing F12 supplemented with FCS. After 72 h, cells were harvested, and lysates were prepared and assayed for total protein, ^{35}S radioactivity, β-hexosaminidase, and NAGLU activity using the flourogenic substrate. In some groups, $Na_2{}^{35}SO_4$-labeled MPS-IIIB fibroblasts were chased with 24-h-conditioned medium from a mass-culture of CHO-K1 cells expressing rNAGLU in the presence or absence of 5 mM M6P.

Preparation of Cell Lysates

Skin fibroblasts and CHO-KI cells were harvested with trypsin-versene (Gibco-BRL) and washed with PBS. Cell lysates were prepared by seven cycles of freeze-thaw in 0.5 M NaCl, 20 mM Tris–HCl, pH 7. Growth medium and cell lysates were clarified by centrifugation. Total cellular protein was quantified using the Bio-Rad protein assay. β-Hexosaminidase activity was determined by using the fluorogenic substrate, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (28).

RESULTS AND DISCUSSION

Purification of Human rNAGLU

Recombinant NAGLU secreted by transfected CHO-K1 cells in the presence of NH_4 Cl was purified from the cell culture medium by a two-step column chromatography using Heparin–Agarose and DEAE-Sephacel. The binding and elution conditions were identical to those used for purification of native NAGLU from human placenta (10), indicating that the recombinant enzyme has similar binding properties and charge.

The microcarrier bead system yielded about 1 mg/L/day when cells had grown to confluency on the beads. This is quite low when compared to another lysosomal enzyme produced by transfected CHO-K1 cells growing on microcarrier beads. For lysosomal acid α-glucosidase a production rate of over 9 mg/L/day had been achieved (15) but expression vector and hence the transcriptional control by a different promotor might have been the decisive factors for the more efficient production of recombinant enzyme. A construct using the same expression vector as for rNAGLU in combination with the cDNA encoding sulfamidase yielded around 2 mg/L/day of recombinant enzyme in transfected CHO-K1 cells (B. Weber, unpublished observations).

TABLE 2

Catalytic Properties of rNAGLU

	K_m (mM)	V_{max} (pmol/min/mg)	Cat. efficiency (10^{-4} L/min/mg)
Fluorogenic substrate − BSA	1.07	4.54×10^5	4.24
Fluorogenic substrate + BSA	5.34	3.97×10^6	7.43
[^{3}H]Disaccharide + BSA	0.0166	4.48×10^4	26.99

Note. Details are given under Materials and Methods section. As disaccharide substrate tritium-labeled GlcNAc-IdOA was used. The enzyme preparation (75 mM NaCl fraction, Fig. 1) consisted mostly of the precursor form of rNAGLU. Catalytic efficiency is the quotient of V_{max} and K_m.

SDS-PAGE showed two bands of approximately 79 and 89 kDa, with the 79 kDa form enriched but not exclusively eluted by 50 mM NaCl from the DEAE-column while the 89 kDa form eluted preferably with higher salt concentrations (Fig. 1). It had not been possible to separate both forms, even if other matrices were used. Previously purified NAGLU had reported molecular masses of 82 kDa for enzyme isolated from human fibroblasts with precursor and intermediate or mature forms ranging from 86 kDa to 77 and 73 kDa (5), whereas for NAGLU purified from human liver a size of 80 kDa was observed (9). NAGLU purified from human kidney carcinoma cells had an apparent molecular weight of 80 kDa (6). A secreted 86-kDa form was observed in the medium of these cells (7) and isolated from urine (8).

The two forms of NAGLU, purified from human placenta with apparent molecular weights of 77 and 80 kDa (10) were differentially eluted with buffer compositions similar to the two forms of recombinant enzyme. The placenta isoforms represent mature enzyme and precursor that differ by 36 amino acids trimmed off the N-terminus. Whereas, the two forms of recombinant enzyme seem to differ in posttranslational modifications of N-glycosylation moieties. Deglycosylation with PNGaseF reduced the apparent molecular weight of both forms to approximately 70 kDa (Fig. 1), indicating that the difference in size of the two rNAGLU forms was due to carbohydrate moieties rather than N-terminal processing of the NAGLU polypeptide.

Both fractions were pooled to immunize a rabbit, generating a polyclonal antiserum that should be directed against both forms of rNAGLU and was used in the immunoprecipitation experiments. The antiserum was also found to recognize precursor, intermediate and mature NAGLU forms found in CHO-K1 cell homogenates (34).

Enzymatic Properties of rNAGLU

The two fractions, enriched in either the 79- or the 89-kDa form of rNAGLU, were treated separately in order to test for differences in enzymatic properties. Both fractions of rNAGLU had identical pH optima of 4.6 toward the fluorogenic substrate with a K_m of 5.34 mM and a V_{max} of 3.97×10^6 pmol/min/mg. Toward a ^{3}H-labeled disaccharide substrate the 75 mM fraction, which consisted mostly of the 89-kDa secreted form of rNAGLU, showed a pH optimum of 4.1, with a K_m of 0.0166 mM and a V_{max} of 4.48×10^4 pmol/min/mg (Table 2). The pH-optima, obtained for *p*-nitrophenyl-α-*N*-acetylglucosamine, of placental, urinary, and liver NAGLU were 4.3, 4.1, and 4.5, respectively (29,30, and 9), indicating that the pH range is comparable for all substrates and enzyme sources tested to date.

It is not clear whether the observed difference in K_m obtained for NAGLU isolated from placenta tissue (30) and rNAGLU (0.55 mM and 5.34 mM, respectively) is due to the recombinant enzyme having a lower substrate affinity, or to the different substrates used to measure catalytic activity. The fluorogenic substrate was reported to have increased sensitivity over the p-nitrophenyl substrate (21) which makes the recombinant enzyme having a lower affinity the more likely explanation. Unlike these artificial substrates, the ^{3}H-labeled disaccharide substrate resembles the structure of natural substrate, heparan sulfate, and thus the lower K_m value of 0.0166 mM is consistent with K_m values observed for other lysosomal enzymes toward their tri- or disaccharide substrates (4, 14). The approximately 1000-fold difference between disaccharide substrate and fluorogenic or *p*-nitrophenyl substrate is consistent with these observations.

Uptake of ^{35}S-Radiolabeled rNAGLU by Skin Fibroblasts

Normal fibroblasts were incubated with ^{35}S-labeled rNAGLU in the absence or presence of 5 mM M6P for 48 h. Recombinant f4S, another lysosomal enzyme known to be efficiently taken up by fibroblasts via a mannose-6-phosphate receptor (MPR)-mediated pathway, was used as a positive control (26). Cells and medium were then harvested and immunoprecipitated using polyclonal antiserum to either rf4S or rNAGLU. Recombinant f4S was secreted from transfected cells as a precursor of a 66-kDa molecule and a 44-kDa mature

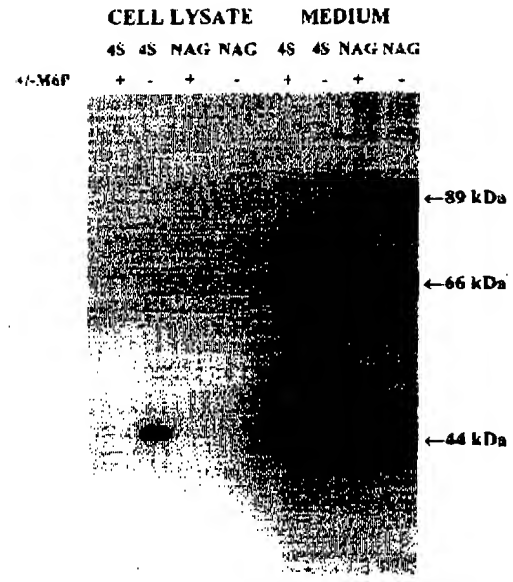

FIG. 2. Uptake of ^{35}S-labeled rf4S and ^{35}S-labeled rNAGLU by skin fibroblasts. Control fibroblasts were incubated with ^{35}S-labeled rf4S (lanes 1–2) or ^{35}S-labeled rNAGLU (rNAG, lanes 3–4) in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of 5 mM M6P for 48 h. Cells and medium were then harvested and immunoprecipitated using polyclonal antiserum to recombinant rf4S (lanes 1–2 and 5–6) or polyclonal antiserum to rNAGLU (lanes 3–4 and 7–8). Precipitates were solubilized, separated by SDS-PAGE, and the gel autoradiographed.

molecule as described previously (26). Direct ^{32}P phosphorylation studies have demonstrated only the precursor form of rf4S to be mannose-6-phosphorylated (31). As expected the mature 44-kDa form of rf4S was taken up into the cells in the absence of M6P while in the presence of M6P the cell surface MPRs were blocked and unable to endocytose the enzyme (Fig. 2). The secreted form of rNAGLU can be immunoprecipitated from the medium but appears not to be taken up by fibroblasts although the medium contained sufficient enzyme to visualize an uptake in the same order of magnitude as rf4S. We can exclude that the polyclonal antiserum failed to detect the 79-kDa mature form since it was able to precipitate intracellular wildtype and mutant forms of rNAGLU (34).

Correction of Storage in NAGLU-Deficient MPS-IIIB Skin Fibroblasts

In order to test whether small amounts of mannose-6-phosphorylated rNAGLU are taken up by MPS-IIIB fibroblasts and remain undetectable by immunoprecipitation, experiments to measure the amount of storage of GAGs in patient fibroblasts were completed. GAGs including heparan sulfate, specifically stored in MPS-IIIB fibroblasts, were radiolabeled by incubation of patient fibroblasts with Na_2 $^{35}SO_4$. Conditioned medium from a mass culture of CHO-K1 cells expressing rNAGLU was placed on the labeled fibroblasts in the presence or absence of 5 mM M6P for 48 h. Results (not shown) were inconclusive and the experiment was repeated allowing uptake for 72 h. The extended chase time resulted in uptake of a small amount of rNAGLU into the cells (Table 3, column 1) which was sufficient to partially correct ^{35}S-labeled GAG storage (Table 3, column 3) while the control enzyme, β-hexosaminidase, remained stable. In the presence of 5 mM M6P no correction is observed indicating endocytosis of rNAGLU by MPRs.

The M6P dependent uptake of rNAGLU increased the intracellular enzyme activity in MPSIII-B fibroblasts to about 3% of the activity measured in normal control cells but this relatively small amount of enzyme was sufficient to reduce the stored GAGs by 75%. This is in good agreement with the observation that less than 10% of normal activity seem to be sufficient for the degradation of lysosomal storage material. Individuals homozygous for the arylsulfatase A pseudodeficiency allele (32) show arylsulfatase A activities of 8% of normal levels but do not develop clinical signs of metachromatic leukodystrophy (33). Although uptake of rNAGLU into MPS-IIIB cells was measured by determining the amount of NAGLU activity present in

TABLE 3

Uptake of rNAGLU and Correction of GAG Storage in MPS-IIIB Skin Fibroblasts

	Intracellular NAGLU (nmol/h/mg)	β-Hexosaminidase (nmol/min/mg)	^{35}S Radioactivity (cpm/mg cell protein)
Normal fibroblasts (control)	6.12 ± 0.14	58.12 ± 1.19	11,975 ± 558
MPS-IIIB fibroblasts (control)	ND	108.79 ± 1.15	170,634 ± 4846
MPS-IIIB fibroblasts + rNAGLU containing medium	0.173 ± 0.05	62.79 ± 0.19	42,234 ± 785
MPS-IIIB fibroblasts + rNAGLU containing medium + 5 mM M6P	ND	68.8 ± 0.24	270,291 ± 1172

Note. Normal or MPS-IIIB skin fibroblasts were metabolically labeled with Na_2 $^{35}SO_4$ for 24 h and then incubated with rNAGLU (8.93 nmol/h/ml, derived from CHO-K1 conditioned medium) in the presence or absence of 5 mM M6P for 72 h. Control cells were incubated with equivalent volumes of medium from nontransfected CHO-K1 cells during the 72-h chase period. Cell lysates were assayed for ^{35}S radioactivity, total protein, β-hexosaminidase activity, or NAGLU activity using the fluorogenic substrate. Results are expressed as the mean (n = 3) ± SD. ND, not detectable.

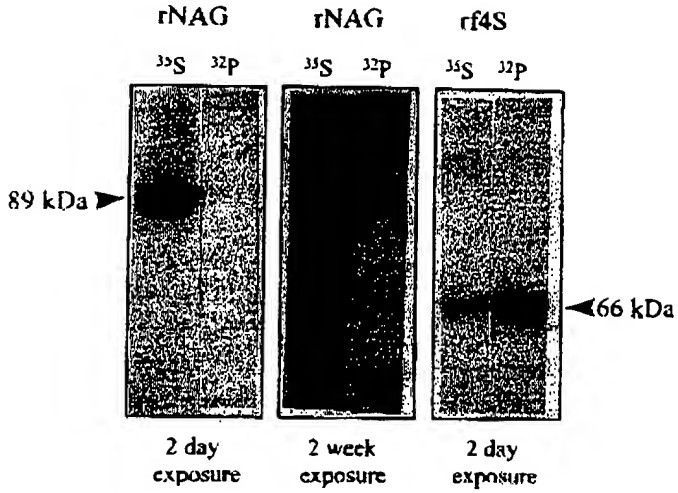

FIG. 3. Specific phosphorylation of rNAGLU and rf4S secreted from CHO-K1 cells. G418-resistant mass-cultures of CHO-K1 cells overexpressing rNAGLU (rNAG) or rf4S were metabolically labeled with $Na_2{}^{32}PO_4$ or EXPRE $^{35}S^{35}S$ protein labeling mix for 24 h in the presence of 5 mM M6P. The medium was then harvested, immunoprecipitated, and analyzed via SDS-PAGE and autoradiography.

these cells, the relative amount of uptake of other lysosomal enzymes may be different for each enzyme and/or cell line. However, the uptake of rNAGLU appears to be much lower than observed for acid α-glucosidase and iduronate-2-sulfatase (13, 15). Taking into account the shorter incubation times and that the uptake of these two enzymes was not dose-dependent, implying that the MPR were saturated (13), the difference is even more striking.

Evidence for Weak Phosphorylation of rNAGLU

The inability to immunoprecipitate rNAGLU taken up by cells (Fig. 2) and the relatively small amount of NAGLU activity able to enter deficient cells via MPR-mediated pathways (Table 2) suggests that the majority of the CHO-K1 secreted rNAGLU is not phosphorylated.

To confirm this ^{32}P-labeled and ^{35}S-labeled NAGLU were immunoprecipitated and analyzed via SDS-PAGE and autoradiography. The specific phosphorylation of recombinant NAGLU secreted form transfected CHO cells was significantly reduced when compared with rf4S (Fig. 3). Nontransfected control CHO-K1 cells contained no labeled immunoprecipitated NAGLU (results not shown), suggesting that the NAGLU enzyme detected by immunoprecipitation corresponds to recombinantly expressed human enzyme and not to endogenous chinese hamster NAGLU.

The observation that NAGLU activity is elevated in the plasma of I-cell patients above that found in normal individuals (34), shows that native intracellular NAGLU is targeted to the lysosome via MPR-mediated pathways and is therefore normally phosphorylated. The expression of rNAGLU in MPSIIIB skin fibroblasts and the resulting corrections of the enzymatic and biochemical storage phenotypes suggests that the intracellular form of rNAGLU is also normally phosphorylated.

It is unlikely that the observed weak phosphorylation of CHO-K1 secreted rNAGLU is due to the cell system used to produce the recombinant enzyme since other lysosomal enzymes were successfully overexpressed in these cells and fully functional in uptake experiments proving their phosphorylation (13–16, 26). A mutation in the expression vector destroying a crucial glycosylation site was excluded by sequencing PCR products amplified from DNA isolated from transfected CHO-K1 cells. Furthermore, the nucleotide sequence of the NAGLU cDNA expressed in this study was obtained independently from two different cDNA libraries representing placenta and testes mRNA (10, 11).

Therefore the weak phosphorylation appears to be a characteristic of the enzyme itself. At present it is not clear whether only a small fraction of newly synthesized rNAGLU is phosphorylated posttranslationally in the first place or whether secreted enzyme is prone to very rapid dephosphorylation. Although the glycosylation pattern of rNAGLU seems to be slightly different when compared to enzyme purified from placenta other characteristics are identical. Since the glycosylation is crucial for the phosphorylation we cannot exclude that the altered pattern is causing the weak phosphorylation or rapid dephosphorylation of the recombinant enzyme while the native enzyme is phosphorylated to the same extend as other lysosomal enzymes. In this case expression of rNAGLU in a different cell system might improve the yield of phosphorylated enzyme. However, weak phosphorylation of secreted rNAGLU expressed in HeLa cells and MPSIIIB skin fibroblasts has also been observed (34), (Yogalingam, unpublished results) further suggesting that the poor phosphorylation is NAGLU-specific rather than expression system-specific.

In addition, recombinant iduronate-2-sulfatase shows no impairment of phosphorylation (13) while overglycosylated and recognition by the enzyme transferring the phosphate residue onto the carbohydrate side chain, UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosamine-1-phosphotransferase, is dependent of the amino acid sequence and protein conformation (35–38). The conformation of rNAGLU, however, seems to be largely unaffected since important features like enzymatic activity and pH optimum are comparable to those of the native enzyme. Since the exchange of crucial amino acids influences the rate of phosphorylation significantly (35), the NAGLU sequence may contain some amino acids that inhibit efficient phosphorylation. In this case the phosphorylated portion would

be transported to the lysosomes while the nonphosphorylated NAGLU fraction is secreted.

Either way, the small degree of phosphorylation that secreted rNAGLU exhibits limits its use considerably since enzyme replacement trials in cells, transgenic animals, or natural animal models for MPSIIIB require the enzyme to be transported via MPR-mediated pathways. Unless alternative efficient delivery systems for unphosphorylated lysosomal enzymes can be utilized, rNAGLU secreted from CHO-K1 cells is of limited use, due to its small degree of phosphorylation.

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